charles river

DRAFT PROTOCOL

Testing Facility Study No. 00459506

An Oral (Drinking Water) Study of the Effects of Trichloroethylene (TCE) on Fetal Heart Development in Sprague Dawley Rats

SPONSOR:
Halogenated Solvents Industry Alliance, Inc.
3033 Wilson Boulevard, Suite 700
Arlington, VA 22201
USA

TESTING FACILITY:
Charles River Laboratories Ashland, LLC
1407 George Road
Ashland, OH 44805
United States

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1. OBJECTIVES

The objective of this study is to determine the potential of trichloroethylene (TCE) to induce cardiac defects in the offspring after maternal exposure from the day after copulation to 1 day prior to expected parturition, to characterize maternal toxicity at the exposure levels tested and to determine a NOAEL (no-observed-adverse-effect level) for maternal and cardiac developmental toxicity.

In addition, plasma concentrations of ~~TCE and~~ TCA (trichloroacetic acid, the primary metabolite of trichloroethylene) will be assessed in maternal and fetal plasma.

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1.1. Study Classification

Study Category:	Developmental and Reproductive Toxicology
Study Type:	Prenatal Development
Study Design:	Parallel
Primary Treatment CAS Registry Number:	79-01-6
Primary Treatment Unique Ingredient ID:	Trichloroethylene
Class of Compound:	Solvent

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual applicable dates will be included in the Final Report.

Animal Arrival:	~~To be determined~~17 Jul 2018
Initiation of Dosing:	~~To be determined~~25 Jul 2018
Completion of In-life:	~~To be determined~~20 Aug 2018
Audited Draft Report:	~~To be determined~~29 Oct 2018

3. GUIDELINES FOR STUDY DESIGN

This study will be conducted in general accordance with the United States Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.3700, Prenatal Developmental Toxicity Study, August 1998, and the Organisation of Economic Cooperation and Development Guidelines (OECD) for the Testing of Chemicals Guideline 414, Prenatal Developmental Toxicity Study, January 2001.

4. REGULATORY COMPLIANCE

This study will be conducted in compliance with the United States Environmental Protection Agency (EPA) TSCA (40 CFR Part 792) Good Laboratory Practice Standards and as accepted by regulatory authorities throughout the European Union (Organization for Economic Cooperation and Development), Japan, and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement. Exceptions to GLPs include the following study elements:

- Test substance characterization will not be conducted according to GLP standards.
- ~~Assessment of concentrations of TCE in maternal and fetal plasma will not be conducted according to GLP standards. A qualified laboratory method developed at Charles River Ashland will be used.~~

Commented [PSC2]: Removed verbiage pertaining to TCE concentration assessment

5. QUALITY ASSURANCE

5.1. Testing Facility

The Testing Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the protocol, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

The Testing Facility QAU contact for this study is indicated below:

Heather L. Johnson, BS, RQAP-GLP
Charles River
1407 George Road
Ashland, OH 44805
Tel: 419.289.8700 x 6874
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6. SPONSOR

Sponsor Representative

Christopher J. Bevan, PhD, DABT
Director, Scientific Programs
Halogenated Solvents Industry Alliance, Inc.
Address as cited for Sponsor.
Tel: (703) 875-0684
Cell: (513) 646-1468
Email: cbevan@hsia.org

Sponsor Study Monitor

Raymond G York, PhD, DABT, ATS, ERT
RG York & Associates LLC
3905 Nicklaus Court
Cincinnati, OH 45245
Cell: (315) 378-9192
Email: ryork2@outlook.com

7. RESPONSIBLE PERSONNEL

Study Director

Prägati Sawhney Coder, PhD, DABT
Director, Developmental and Reproductive Toxicology
Address as cited for Testing Facility
Tel: (419) 289-8700
Email: pragati.coder@crl.com

Alternate Contact

Mark T. Herberth, BS, LATG
Senior Research Scientist, Developmental and Reproductive Toxicology
Address as cited for Testing Facility
Tel: (419) 289-8700
Email: mark.herberth@crl.com

Commented [PSC3]: Added Mark as an alternate contact. Mark is a Senior Research Scientist in my group and will serve as backup for technical questions from the staff, in the event that I am unavailable.

Management Contact

Donald G. Stump, PhD, DABT
Senior Director, Toxicology
Address as cited for Testing Facility
Tel: (419) 289-8700
Fax: (419) 287-3650
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Individual Scientists (IS) at the Testing Facility

Dose Formulation Analysis	Shiladitya Sen, PhD Senior Research Scientist, Analytical Chemistry Address as cited for Testing Facility Tel: (419) 289-8700 Fax: (419) 287-3650 Email: Shiladitya.Sen@crl.com

Plasma Analysis	Joelle Lucarell, PhD Research Scientist II, Bioanalytical Chemistry Address as cited for Testing Facility Tel: (419) 289-8700 Fax: (419) 287-3650 Email: Joelle.Lucarell@crl.com

Each IS is required to report any deviations or other circumstances that could affect the quality or integrity of the study to the Study Director in a timely manner and the Study Director will provide notification to the Sponsor Representative within 24 hours. Each IS will provide a report addressing their assigned phase of the study, which will be included as an appendix to the Final Report. The phase report will include the following:

- A listing of critical computerized systems used in the conduct and/or interpretation of the assigned study phase.

8. TEST SUBSTANCE, POSITIVE CONTROL SUBSTANCE AND VEHICLE DATA

8.1. Test Substance

8.1.1. Identification

Trichloroethylene (TCE) (CAS No. 79-01-6) ≥99% and scavenger-free

Purchased from Spectrum Chemical Manufacturing Corp. (T1115 reagent grade, or equivalent).

8.1.2. Characterization

Lot numbers, purity, stability, and storage conditions will be provided by the Supplier/Manufacturer, documented in the study records and included in the Final Report.

8.1.3. Storage Conditions

In a room with controls set to maintain 18°C to 24°C, protected from light.

8.1.4. Physical Description

To be documented by Charles River.

8.1.5. Reserve Samples

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Reserve samples of the test substance will be taken in accordance with Charles River Standard Operating Procedures and stored in the Charles River Archives ~~indefinitely, unless otherwise specified~~.

8.1.6. Personnel Safety Data

A ~~Material~~ Safety Data Sheet (MSDS) is to be provided by the Supplier/Manufacturer. Standard safety precautions will apply.

8.1.7. Test Article Disposition

With the exception of the reserve sample for each batch of test article (if applicable), all neat test article remaining at study completion will be discarded appropriately.

8.2. Positive Control Substance

8.2.1. Identification

all-*trans* Retinoic Acid ≥98% by HPLC (CAS No. 302-79-4)

Purchased from Sigma-Aldrich, Inc. (R2625, or equivalent)

8.2.2. Characterization

Lot numbers, purity, stability, and storage conditions will be provided by the Supplier/Manufacturer, documented in the study records and included in the Final Report.

8.2.3. Storage Conditions

In a freezer, set to maintain -20°C, protected from light.

8.2.4. Physical Description

To be documented by Charles River

8.2.5. Reserve Samples

Commented [PSC5]: Minor edits per current CRL protocol template.

Reserve samples of the positive control substance will be taken in accordance with Charles River Standard Operating Procedures and stored in the Charles River Archives ~~indefinitely, unless otherwise specified~~.

8.2.6. Personal Safety Data

A ~~Material~~ Safety Data Sheet (~~M~~SDS) is to be provided by the Supplier/Manufacturer. Standard safety precautions will apply.

8.3. Vehicle (for Drinking Water Formulations)

8.3.1. Identification

Reverse osmosis-purified water

8.3.2. Characterization

Water used on-site is subject to routine monitoring as indicated in SOP A-067. Standard safety precautions will apply.

8.4. Vehicle (for Positive Control Formulations)

8.4.1. Identification

Soybean oil (CAS No. 8001-22-7)

Purchased from Sigma-Aldrich, Inc. (S7381 dietary grade, or equivalent)

8.4.2. Characterization

Lot numbers, purity, stability, and storage conditions will be provided by the Supplier/Manufacturer, documented in the study records and included in the Final Report.

9. PREPARATION AND ANALYSIS OF TEST AND POSITIVE CONTROL SUBSTANCE FORMULATIONS

9.1. Test Substance Formulations

Commented [PSC6]: Section updated per changes successfully incorporated during the Feasibility study 00459507

9.1.1. Method and Frequency of Preparation

Based on the physical characteristics of the test substance, appropriate methods will be used to ensure the best possible formulations of the test substance in the vehicle. Test substance formulations will be prepared daily, in a closed system, under amber light, without sonication, and stored and transported in the same closed system amber formulation bottles (for light protection). Each amber formulation bottle will be purged with nitrogen, sealed with a foil liner and silicone septum fitted with a fabricated siphon valve system built at Charles River Ashland.

All formulation batches will be prepared at volumes large enough to minimize headspace. The 500 and 1000 ppm concentrations will be prepared the day prior to dosing and stirred overnight at room temperature for at least 24 hours. The 0.25 and 1.5 ppm concentrations will be prepared via dilution of higher concentrations on the day of dose administration. Test substance formulations will be stored at room temperature (18°C to 24°C) following preparation and until transfer into drinking water bottles for administration to study animals.

For transfer into drinking water bottles, the inlet valve on each formulation bottle will be connected to a nitrogen source to allow nitrogen to displace dosing formulations that are removed via the outlet valve. Purging of any headspace with nitrogen will help reduce volatilization of TCE and ensures that residual water formulations do not come in contact with ambient air. Drinking water bottles will be filled by allowing the water to flow along the inner wall, to reduce splashing, bubbling and volatilization of TCE.

~~Special precautions will be taken to ensure that dosing formulations are prepared and transported in a closed system, and all closed formulation containers will be purged with nitrogen. All formulations will be prepared at volumes that minimize headspace in the preparation vessels. All dose concentrations at or above 100 ppm will be prepared the day prior to dosing and stirred overnight at room temperature for at least 24 hours. Concentrations below 100 ppm will be prepared via dilution of higher concentrations on the day of dose administration. Test substance~~

formulations will be stored at room temperature (18°C to 24°C) following preparation and until transfer into drinking water bottles for administration to study animals. Test substance formulations will be transferred into drinking water bottles via a sealed valve system built at Charles River Ashland.

Any procedures not covered by SOPs required for formulation will be approved by the Study Director and included in the study records.

The Study Director or designee will visually inspect the test substance formulations prior to initiation of dosing. This visual inspection will be performed to ensure that the formulations are visibly homogeneous and acceptable for dosing.

9.1.2. Solubility and Stability of Test Substance in Drinking Water Formulations

Test substance formulations in drinking water will be analyzed using a method previously developed and validated at Charles River Ashland.[1] Solubility and stability of the test substance in the vehicle following room temperature (18°C to 24°C) storage for at least 24 hours, and following frozen (purged with nitrogen, -10°C to -20°C) storage, at the range of concentrations being used on the current study was previously established.[NOTEREF _Ref504930085 \h * MERGEFORMAT] Therefore, solubility and stability and homogeneity/solubility of test substance formulations will not be assessed on the current study.

9.1.3. Concentration of Test Substance in Drinking Water Formulations

Concentration of test substance in "as-delivered" dosing formulations, including the vehicle control, will be assessed on the 1st, 2nd, 3rd, 7th, 12th, 15th, 22nd and last batch of drinking water formulations. For analytical purposes, the last batch will be the last day all prepared batches (at all concentrations) are used for administration to animals (i.e. taking into consideration breeding stagger). Samples for possible concentration assessment will also be collected from all remaining daily batches, purged with nitrogen, and stored in a freezer set to maintain a target of -20°C.

Sampling, processing and analysis of prepared drinking water formulations will be conducted on the day of distribution prior to transfer into drinking water bottles for administration to study animals according to the table below. For preparations scheduled for analysis, samples will be processed and analyzed as soon as possible following collection.

Test Substance Formulation Sampling Scheme

Group(s)	Time of Sampling	Formulation Container	Sample Scheme and Volume[a]	Formulation Preparation Number(s)[b]
1, 3-6	Time of Prep (Closed System)	Amber Formulation Bottle	2 x 10 mL	First, 2nd, 3rd, 7th, 12th, 15th, 22nd and Last
1, 3-6	Time of Dispensation (Open System)	Amber Drinking Water Bottle	2 x 10 mL	First, 2nd, 3rd, 7th, 12th, 15th, 22nd and Last
1, 3-6	24h Post-Dispensation (Open System)	Amber Drinking Water Bottle	(2 x 10 mL) x 3 bottles	First, 2nd, 3rd, 7th, 12th, 15th, 22nd and Last

[a] All samples will be collected from the middle stratum, into amber glass auto-sampler vials with rubber

stoppers, and crimped tops.

[b] For analytical purposes, the last batch will be the last day all prepared batches (at all concentrations) are used for administration to animals (i.e. taking into consideration breeding stagger).

All samples will be collected in amber glass auto-sampler vials with rubber stoppers and crimped tops. Following acceptance of each set of analytical results, by the study director and the Sponsor Representative, any prior unanalyzed batches up until that point will be discarded appropriately (e.g. following analysis of the 7th batch, and acceptance of the analytical results, samples from the 4th, 5th and 6th (unanalyzed) batches will be discarded.

) For analytical purposes, the last batch will be the last day all prepared batches (at all concentrations) are used for administration to animals (i.e. taking into consideration breeding stagger). Sampling, processing and analysis of prepared drinking water formulations will be conducted on the day of distribution prior to transfer into drinking water bottles for administration to study animals. Two 1.0 mL samples will be collected from the middle of the vehicle control and each test substance drinking water formulation for assessment of the concentration of the test substance in the formulations. Samples will be processed and analyzed as soon as possible following collection.

No additional sampling or analysis of drinking water formulations will be conducted following transfer to an open system (i.e. drinking water bottles) with the exception of 24-Hour loss monitoring as described below. For consistency and ease of reporting, concentrations for each dose group in the protocol and report tables will be referred to by the initial (target) concentration as has been used in previously published reports.[2,3] Calculated compound consumption will be based on analytically confirmed concentrations at each assessment interval. The target acceptance criteria for concentration assessment of TCE in drinking water formulations will be mean concentrations within 100% ± 20% (80-120%) of the target concentration. However, because of the volatility of the test substance, it is recognized that this acceptance criteria may not be achievable for each formulation and concentration. If any formulations do not met acceptance criteria, the impact of the out-of-specification results will be addressed in the report.

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24-hour Loss Monitoring – Samples collected 24-hours post-dispensation will be collected from "used" water bottles in the animal room and will be processed and analyzed for concentration assessment as soon as possible following collection. Because of the open system and the volatility of the test substance, measured concentrations will be reported as-is i.e. target acceptance criteria will not apply to 24-hour loss monitoring samples. Loss of TCE at each concentration, will be calculated by averaging of the three sampled bottles and comparison against corresponding Time Zero concentrations (measured concentrations prior to transfer into drinking water bottles) and will be reported as a Percent 24-Hour Loss for each concentration.

In order to determine how much TCE is lost from drinking water formulations over the course of 24-hours in an open system (i.e. drinking water bottles), formulations from the first batch will be sampled following approximately 24-hours of administration to animals. Two 1.0 mL samples will be collected from the middle of the vehicle control, and from 3 randomly selected bottles with test substance drinking water formulation (at each concentration). Samples will be taken

from "used" water bottles in the animal room and will be processed and analyzed for concentration assessment as soon as possible following collection. Because of the open system and the volatility of the test substance, measured concentrations will be reported as-is i.e. target acceptance criteria will not apply to 24-hour loss monitoring samples. Loss of TCE at each concentration, will be calculated by averaging of the three sampled bottles and comparison against corresponding Time Zero concentrations (measured concentrations prior to transfer into drinking water bottles) and will be reported as a Percent 24-Hour Loss for each concentration.

The final analytical report will be incorporated as an appendix to the Charles River final report.

9.2. Positive Control Substance

9.2.1. Method and Frequency of Preparation

Based on the physical characteristics of the positive control substance, appropriate methods will be used to ensure the best possible formulations in the vehicle, soybean oil, which may be warmed to ensure solubilization, if necessary. Positive control substance formulations will be prepared under amber light and stored and transported in small amber aliquot bottles for light protection. Positive control substance formulations will normally be prepared approximately weekly, divided into aliquots for daily dispensation, purged with nitrogen and stored in a freezer, set to maintain a target of -20ºC. The positive control formulations will be thawed for each day of administration, and dispensed after remixing for a minimum of 30 minutes using a magnetic stirrer. Positive control formulations will be stirred continuously during dosing.

Any procedures not covered by SOPs required for formulation will be added to the protocol by protocol amendment and presented in the final report of this study.

9.2.2. Concentration of Positive Control Substance in Soybean Oil Formulations

Positive control formulations in the vehicle, soybean oil, will not be assessed for solubility, concentration, homogeneity, or stability. All-*trans* retinoic acid (RA) is a commercially available drug substance that will be prepared according to package specifications. It is a well characterized developmental toxicant that has been previously demonstrated to result in heart malformations in this strain of rat.[4]

Sampling of positive control substance dosing formulations will be conducted for For future possible concentration assessments will be conducted according to the table below. of Samples will be purged with nitrogen and concentration of the positive control substance in dosing formulations, duplicate 1.0 mL samples will be collected from the middle strata on the first and last day of use for each batch of dosing formulations. Samples will be purged with nitrogen and stored in a freezer set to maintain a target of -20ºC.

Positive Control Substance Formulation Sampling Scheme

Group(s)	Time of Sampling	Formulation Container	Sample Scheme and Volume	Formulation Preparation Number(s)
2	Time of Prep	First Aliquot	2 x 1 mL	All

2	Time of Dispensation	Last Aliquot	2 x 1 mL	All

If samples are analyzed, the final analytical report will be incorporated as an appendix to the Charles River final report.

Following completion of the in-life phase of the study and the acceptance of study results by the study director and the Sponsor Representative, any unanalyzed samples will be discarded appropriately (i.e. samples will not be archived, but will be discarded prior to issuance of the final report).

10. TEST SYSTEM

Species:	Rat
Strain:	Sprague Dawley Crl:CD(SD)
Condition:	Naïve, Nonpregnant
Source:	Charles River Laboratories, Inc. (Raleigh, North Carolina)
Number of Males Ordered:	A sufficient number of sexually mature untreated resident males of the same strain and source will be purchased to induce pregnancies.
Number of Females Ordered:	210
Target Age at the Initiation of Breeding:	80 to 120 days at the initiation of breeding
Target Weight on Gestation Day 0:	A minimum of 220 g

Animals not assigned to the study will be transferred to the animal colony or will be euthanized by carbon dioxide inhalation and the carcasses discarded. The actual age and weight of animals received will be listed in the Final Report.

10.1. Identification System

A permanent animal number will be assigned to each individual animal. Each animal will be identified using a subcutaneously implanted electronic identification microchip (BMDS system). The microchip will be the primary means to uniquely identify animals assigned to study. Individual cage cards will be affixed to each cage and will display at least the animal number, group number, dosage level, study number, and sex of the animal.

Replacement microchips may be implanted as necessary throughout the course of the study. An ear tag may be used as the alternate unique identifier.

10.2. Justification for Selection

The purpose of this study is to replicate the findings of Dawson et al.[NOTEREF _Ref511308716 \h * MERGEFORMAT] and Johnson et al.[NOTEREF _Ref504935597 \h * MERGEFORMAT] In these studies it was

reported that there was an increase in cardiac malformations in the fetuses of pregnant female Sprague Dawley rats administered TCE in drinking water.

This species and strain of rat has been recognized as appropriate for developmental toxicity studies. Charles River has historical data on the background incidence of fetal malformations and developmental variations in this species from the same strain and source. This animal model has been proven to be susceptible to the effects of developmental toxicants

10.3. Number of Study Animals

The number of animals is based on the US EPA Health Effects Test Guidelines OPPTS 870.3700, Prenatal Development Toxicity Study, August 1998 and the OECD Guidelines for the Testing of Chemicals: Guideline 414, Prenatal Developmental Toxicity Study, January 2001, which recommend evaluation of approximately 20 females with implantation sites at necropsy. Given the possibility of nongravid animals, unexpected deaths, or treatment-related moribundity and/or mortality, 25 females/group is an appropriate number to obtain a sample size of 20 females at termination.

The number of animals assigned to the toxicokinetic phase (4 females/group) is also based on the possibility of nongravid animals, unexpected deaths, or treatment-related moribundity and/or mortality; this is an appropriate number of animals to obtain at least 3 blood samples per time point.

11. SPECIFIC ANIMAL MAINTENANCE SCHEDULE

11.1. Animal Receipt and Acclimation

Each rat will be inspected by a qualified technician upon receipt. Rats judged to be in good health and suitable as test animals will be immediately placed in acclimation for a minimum of 7 days. All rats will be initially weighed, permanently identified with a microchip, and will receive a detailed clinical observation. During the acclimation period, each rat will be observed twice daily for changes in general appearance and behavior. Body weights will be recorded prior to the initiation of breeding. Prior to the start of breeding, those rats judged to be suitable test subjects will be identified.

During social housing, some observations (e.g., fecal observations) may not be attributable to an individual animal. In these instances, observations will be recorded in a separate computer file for the social group.

11.2. Animal Housing

Female rats will be housed, 2-3 per cage, in clean solid-bottom cages with bedding material (Bed O'Cobs® or other suitable material) for at least 3 days following receipt in an environmentally controlled room. Following positive signs of mating, each female will be individually housed in clean, solid-bottom cages with bedding material (Bed O'Cobs® or other suitable material) until euthanasia. Animals may be temporarily separated for protocol-specified

activities and this will be documented in the study records. In addition, animals may be individually housed due to incompatible behavior with a cage mate(s) or for health monitoring purposes requested by the veterinarian. Animals whose cage mate(s) are removed from study (morbidity, unscheduled death, etc.) will not be re-paired but will remain individually housed for the remainder of the study.

The cages will be subjected to routine cleaning at a frequency consistent with maintaining good animal health and Charles River Standard Operating Procedures. The facilities at Charles River Ashland are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Individual housing of presumed pregnant females is required to adequately monitor the health of these females by allowing collection of individual food consumption and appropriate identification of cage observations in the event of abortion or early delivery

11.3. Environmental Conditions

Controls will be set to maintain temperature at 73 ± 5°F (23 ± 3°C) and relative humidity at 50 ± 20%. Temperature and relative humidity will be monitored continuously. Data for these 2 parameters will be scheduled for automatic collection on an hourly basis. Fluorescent lighting controlled by light timers will provide illumination for a 12-hour light/dark photoperiod. The ventilation rate will be set at a minimum of 10 room air changes per hour, 100% fresh air.

11.4. Drinking Water

Cage banks will not be connected to the automated watering system. Reverse osmosis-purified water (with test substance added during the treatment period for animals assigned to Groups 3-6) will be available *ad libitum* via amber glass water bottles with metal sipper tubes. Bottles will be checked daily for spillage and supplemented as necessary and the occurrence of spillage will be documented. During the treatment period, bottles will be changed daily. The municipal water supplying the laboratory is analyzed according to Charles River Ashland SOPs on a routine basis to ensure that contaminants are not present in concentrations that would be expected to affect the outcome of the study.

11.5. Basal Diet

PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 will be offered *ad libitum* during the study. Periodic analyses of the certified feed are performed by the manufacturer to ensure that heavy metals and pesticides are not present at concentrations that would be expected to affect the outcome of the study. Results of the analyses are provided to Charles River by the manufacturer. Feeders will be changed and sanitized once per week.

11.6. Environmental Enrichment

Enrichment devices will be provided to each animal for environmental enrichment beginning during acclimation, and continuing throughout the course of the study.

12. EXPERIMENTAL DESIGN

12.1. Breeding Procedure

At the conclusion of the acclimation period, female rats judged to be suitable test subjects and meeting acceptable body weight requirements will be cohabitated with untreated resident male rats (1:1) of the same strain and source in solid-bottom cages for mating. Detection of mating will be confirmed by the appearance of a vaginal copulatory plug or by evidence of sperm in a vaginal lavage. Vaginal lavages will be performed daily during the mating period until evidence of mating is observed. After confirmation of mating, the female will be returned to an individual solid bottom cage (assigned to a group), and the day will be designated as day 0 of gestation.

12.2. Animal Selection and Randomization

Mated females will be assigned to groups using a WIL Toxicology Data Management System (WTDMS™) computer program which assigns animals based on stratification of Gestation Day 0 body weights into a block design to 1 vehicle control group, 1 positive control group and 4 test substance groups of 25 rats each for the prenatal developmental (Main) phase. For the exposure assessment (Exp.) phase, the vehicle control and 4 test substance groups will consist of 4 rats each.

Following the initiation of dosing, it may be necessary to add individual animal(s) (due to animals being found dead, euthanized *in extremis*, exhibiting abnormal clinical signs, reduced food consumption, body weigh losses, or dosing errors). Individual animals that are added to the study will be selected from the remaining unassigned mated animals, and assigned arbitrarily (not computer randomized) to the study based on comparable body weights (if possible) with respect to the animal(s) previously assigned to the study. The reason(s) for adding the animal(s) will be appropriately documented in the study records. The cut-off gestation age for adding animals to study is Gestation Day 1 for the vehicle control and test substance groups and Gestation Day 6 for the positive control group.

12.3. Organization of Test Groups, Dosage Levels, and Treatment Regimen

12.3.1. Rationale for Dose Selection

The dosage levels were selected based on previous published reports assessing fetal heart development in Sprague Dawley rats[NOTEREF _Ref504935597 \h],[NOTEREF _Ref461106285 \h * MERGEFORMAT],[5] and were provided by the Sponsor Representative after consultation with the Charles River Study Director.

The positive control substance, RA, is a well characterized developmental toxicant that has been previously demonstrated to result in heart malformations in this strain of rat. The dosage level of RA was also selected based on previously published reports.[NOTEREF _Ref461106285 \h * MERGEFORMAT]

12.3.2. Organization of Test Groups

The following table presents the study group arrangement.

Study Design

Group Number	Test Substance	Dosage Level (mg/kg/day)	Dose Concentration	Dose Volume (mL/kg)	Route of Administration	Number of Females	
						Main	Exp.
1	Vehicle control	0	0 ppm	NA	Drinking Water	25	4
2	RA	15	3 mg/mL	5	Gavage	25	0
3	TCE	a	0.25 ppm	NA	Drinking Water	25	4
4	TCE	a	1.5 ppm	NA	Drinking Water	25	4
5	TCE	a	500 ppm	NA	Drinking Water	25	4
6	TCE	a	1000 ppm	NA	Drinking Water	25	4

a Dosage levels for the drinking water groups (i.e. mean amount of TCE received by each group of rats) will be calculated upon completion of the study based on mean water consumption of each group and target concentration of the test substance in water formulations. For consistency and ease of reporting, concentrations for each dose group will be referred to by the initial target concentration as has been used in previously published reports.[NOTEREF _Ref504935597 \h * MERGEFORMAT]

12.3.3. Route and Rationale of Test Article Administration

The route of administration of the test substance will be oral (drinking water) as this is a potential route of exposure for humans.

The positive control substance, RA, will be administered via oral (gavage) as that route of exposure has been demonstrated to elicit a positive response.[NOTEREF _Ref461106285 \h * MERGEFORMAT]

12.3.4. Treatment Regimen - Test and Positive Control Substances

Vehicle control or test substance drinking water formulations will be offered *ad libitum* from Gestation Day 1 through euthanasia (scheduled for Gestation Day 21). Water formulations will be supplied fresh on a daily basis, within ± 2-3 hours from the previous day.

The positive control substance will be administered as a single daily dose from Gestation Day 6 through 15, inclusively (Group 2 only). This is the standard dosing regimen for a prenatal developmental toxicity study and is expected to elicit a positive response.[NOTEREF _Ref461106285 \h * MERGEFORMAT] All rats will be dosed at approximately the same time each day.

The positive control group (Group 2) will receive vehicle control drinking water formulations *ad libitum* from Gestation Day 1 through euthanasia. Water formulations will be supplied fresh on a daily basis.

12.3.5. Method of Test Article Administration

Control and treated drinking water formulations will be offered *ad libitum* in amber glass water bottles with metal sipper tubes. Water bottles will be changed and sanitized daily, and drinking water formulations will be supplied fresh on a daily basis.

The positive control substance will be administered orally by gavage (Group 2 only) using appropriately sized disposable plastic feeding tubes (Instech Laboratories, Plymouth Meeting, PA). The dose volume will be 5 mL/kg. Formulations will be stirred continuously at room temperature for the duration of the dosing procedure.

12.3.6. Adjustment of Dose Volumes

The test substance will be administered as a constant concentration (ppm) in water.

For the positive control substance treated group (Group 2), individual dosages will be calculated on the most recent body weight to provide the proper mg/kg/day dosage.

13. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS

13.1. Viability Observations

Each rat will be observed twice daily for moribundity and mortality, once in the morning and once in the afternoon from Gestation Day 0 until euthanasia.

13.2. Maternal Observations during Gestation

Detailed clinical observations will be recorded daily prior to administration of new daily water bottles. Mortality and all signs of overt toxicity will be recorded on the day observed. The observations shall include, but are not limited to, evaluations for changes in appearance of skin and fur, eyes, mucous membranes, respiratory and circulatory system, autonomic and central nervous systems, somatomotor activity, and behavior. All animals will also be observed on the day of necropsy and any findings will be recorded.

For the positive control substance treated group (Group 2 only), individual clinical observations will be recorded approximately 1 hour following each dose administration for findings that are potentially related to treatment or that might change before the next scheduled observation. Additional observations may be necessary and will be documented in the study records.

13.3. Body Weights

Individual body weights will be recorded on Gestation Days 0-21 (daily) for animals assigned to the main and exposure assessment phases.

13.4. Water Consumption

Individual water consumption (by weight) will be recorded on Gestation Days 0-21 (daily) for animals assigned to the main and exposure assessment phases.

The mean amount of TCE received by each group of rats (test substance consumption) will be calculated upon completion of the study based on mean water consumption of each group and the target concentration of the test substance in water formulations. For consistency and ease of reporting, concentrations for each dose group will be referred to by the initial target

concentration as has been used in previously published reports.[NOTEREF _Ref504935597 \h * MERGEFORMAT]

13.5. Food Consumption

Individual food consumption will be recorded on Gestation Days 0-21 (daily) for animals assigned to the main phase. Food intake will be reported as g/animal/day and g/kg/day for each corresponding body weight interval of gestation.

Food consumption will not be recorded for animals assigned to the exposure assessment phase.

13.6. Deaths and Animals Euthanized in Extremis

Females not surviving until the scheduled euthanasia will be necropsied (as soon as possible upon discovery) and cause of death recorded, if possible. Rats not expected to survive to the next observation period (moribund) will be euthanized by carbon dioxide inhalation. The cranial, thoracic, abdominal, and pelvic cavities will be opened and the organs examined. The number and location of implantation sites and viable fetuses will be recorded. Corpora lutea will also be counted and recorded. Uteri which appear nongravid by macroscopic examination will be opened and placed in 10% ammonium sulfide solution for detection of early implantation loss.[6] Gross lesions will be preserved in 10% neutral-buffered formalin for possible future histopathologic examination. Carcasses from adult animals will be discarded. Viable fetuses will be euthanized by a subcutaneous injection of sodium pentobarbital in the scapular region. Recognizable fetuses will be examined externally and preserved in 10% neutral-buffered formalin.

Animals dying or euthanized *in extremis* (by carbon dioxide inhalation) that are assigned to the exposure assessment phase will have pregnancy status determined (by ammonium sulfide, if necessary). Viable fetuses will be euthanized by a subcutaneous injection of sodium pentobarbital in the scapular region. Carcasses of the dams and fetuses will be discarded.

13.7. Premature Deliveries

Females that deliver prematurely will be euthanized by carbon dioxide inhalation that day. The thoracic, abdominal, and pelvic cavities will be opened and the organs examined. The number and location of former implantation sites and viable fetuses will be recorded. Corpora lutea will also be counted and recorded. Gross lesions will be preserved in 10% neutral-buffered formalin for possible future histopathologic examinations. Carcasses from adult animals will be discarded. Viable fetuses or pups will be euthanized by a subcutaneous (scapular region) or intraperitoneal injection of sodium pentobarbital (as appropriate). Recognizable fetuses or pups will be examined externally and preserved in 10% neutral buffered formalin. Recognizable fetuses or pups aborted on GD 21 will be examined according to the fetal examination section (Section 15.2), if possible.

Females that deliver prematurely that are assigned to the exposure assessment phase will be euthanized by carbon dioxide inhalation that day and identified as gravid. Viable pups will be

euthanized by an intraperitoneal injection of sodium pentobarbital. Carcasses of the dams and pups will be discarded.

14. LABORATORY EVALUATIONS (EXPOSURE ASSESSMENT PHASE)

14.1. Intervals

Dams: Gestation days 8, 12 and 21

Fetuses: Gestation Day 21

Commented [PSC7]: Removed verbiage pertaining to TCE concentration assessments throughout. Since TCA is not volatile, we don't need to use vacutainer tubes. Also, we are still checking on availability of Amber EDTA tubes, if not, samples will be protected from light be storage and transfer in closed coolers and with the use of aluminum foil.

14.2. Blood Collection Time Points

Dams (Gestation Day 8 and 12): A single blood samples will be collected from each dam between 0830 and 0930 hours.

Dams and Fetuses (Gestation Day 21): A single blood sample will be collected from each dam just prior to euthanasia. Immediately following blood collection, each dam will be euthanized by carbon dioxide inhalation and uteri which appear gravid by macroscopic examination will be removed for fetal blood collection. For any dams that initiate parturition prior to blood collection, blood samples will be still be collected, as scheduled on Gestation Day 21/Lactation Day 0. Delivered pups (Postnatal Day 0) belonging to these females will be bled in the same manner as the Gestation Day 21 fetuses.

14.3. Number of animals

Dams: Four (4) females/group assigned to the exposure assessment phase.

Fetuses: Four (4) litters ~~(pooled by litter)~~ per group from dams assigned to the exposure assessment phase. Blood will be pooled by litter, without regard to fetal sex.

14.4. Method/Route of Collection

Dams: via the jugular vein using the hand-held restraint method.

Fetuses: via cardiac puncture under isoflurane anesthesia. Delivered pups (Postnatal Day 0) belonging to any females that deliver prior to blood collection will be bled in the same manner as the Gestation Day 21 fetuses.

14.5. Target Blood Volume

Dams: 0.5 mL/animal/time point; samples will be transferred as rapidly as possible from the collection syringe into pre-chilled, uniquely labeled ~~amber vacutainer~~ tubes. Samples will be protected from light, to the extent possible.

Fetuses: As much blood as possible; blood will be pooled by litter regardless of sex. Samples will be transferred as rapidly as possible from the collection catheter/syringe into pre-chilled, uniquely labeled ~~amber vacutainer~~ tubes. Samples will be protected from light, to the extent possible.

14.6. Anticoagulant

Lithium HeparinK2 EDTA (amber vacutainer tubes)

14.7. Sample Handling and Plasma Preparation

Samples will be kept on wet ice, protected from light, following blood collection and through centrifugation, plasma collection, and storage. All samples will be centrifuged (approximately 3000 rpm [approximately 2056xg] for approximately 10 min) at approximately 4°C. Samples will be processed under amber light.

14.8. Aliquots

The maximum amount of plasma will be recovered and plasma will be transferred into new, uniquely-labeled amber polypropylene tubes.

14.9. Label Information

Samples, and/or accompanying paperwork, will include study number, dose group, animal number, Gestation Day interval, number of pups (in pooled samples), sample type, date and time of blood collection.

14.10. Sample Storage and Transfer

Commented [PSC8]: Removed verbiage pertaining to TCE concentration assessment

Also combined the Sample Storage and Sample Transfer Sections into 1 section, per current CRL protocol template.

Maternal and fetal Plasma plasma samples will be stored in a freezer set to maintain a target of -70°C until transferred to the Charles River Bioanalytical Chemistry Department for analysis for the assessment of TCA concentrations using a method being developed and validated on a concurrent study.[7] The time and date that the samples are placed in the freezer will be recorded.

Any remaining samples kept at Charles River will be discarded following acceptance of the bioanalytical results by the Study Director.

The plasma analysis report will be included as an appendix to the Charles River final report.

14.11. Disposition of Animals/Laparotomy

All exposure assessment phase rats will be euthanized by carbon dioxide inhalation following the last blood collection (GD 21). Uteri which appear gravid by macroscopic examination will be removed immediately for fetal blood collection and the dams will be identified as gravid. Uteri which appear nongravid by macroscopic examination will be opened and placed in 10% ammonium sulfide solution for detection of early implantation loss.[NOTEREF _Ref461106913 \h * MERGEFORMAT] Following blood collection, fetuses will be euthanized by decapitation. Carcasses of the dams and fetuses will be discarded without further examination.

~~14.12. Sample Transfer for Plasma Analysis~~

~~Plasma samples, an inventory list and documentation of actual blood collection times for each animal, will be transferred to the Charles River Bioanalytical Chemistry Department for assessment of TCE and TCA concentrations in maternal and fetal samples.~~

~~Any remaining samples kept at Charles River will be discarded following acceptance of the bioanalytical results by the Study Director.~~

~~The plasma analysis report will be included as an appendix to the Charles River final report.~~

~~14.13.~~ 14.12. **Exposure Assessment**

Commented [PSC9]: Removed verbiage pertaining to TCE concentration assessment

Plasma concentrations of ~~TCE and~~ TCA in maternal and fetal samples will be summarized and presented in the main report text. Based on the limited blood sampling, the analysis of exposure data will be limited to mean concentrations, by group, and maternal and fetal concentration ratios.

15. TERMINAL PROCEDURES – GESTATION DAY 21 (PRENATAL DEVELOPMENT PHASE)

15.1. Laparohysterectomy and Macroscopic Examination

Laparohysterectomy and macroscopic examinations will be performed blind to treatment group. All surviving rats will be euthanized by carbon dioxide inhalation on Gestation Day 21. The thoracic, abdominal, and pelvic cavities will be opened and the organs examined. The uterus of each dam will be excised and its adnexa trimmed. Corpora lutea will be counted and recorded. Gravid uterine weights will be obtained and recorded. The uterus of each dam will be opened and the number of viable and nonviable fetuses, early and late resorptions, and total number of implantation sites will be recorded, and the placentae will be examined. The individual uterine distribution will be documented using the following procedure: all implantation sites, including early and late resorptions, will be numbered in consecutive fashion beginning with the left distal uterine horn, noting the position of the cervix and continuing from the proximal to the distal right uterine horn. Uteri which appear nongravid by macroscopic examination will be opened and placed in a 10% ammonium sulfide solution for detection of early implantation loss.[NOTEREF _Ref461106913 \h * MERGEFORMAT] Maternal tissues will be preserved for future histopathologic examination in 10% neutral-buffered formalin only as deemed necessary by the gross findings. Representative sections of corresponding organs from a sufficient number of controls will be retained for comparison, if possible. The carcasses will be discarded.

15.2. Fetal Examination

Fetal examinations will be conducted without knowledge of treatment group. All fetuses will receive an external examination. **Internal (visceral) examination will be limited to an examination of the heart and great and major blood vessels only.** Representative photographs of all cardiac and great and major blood vessel malformations, as appropriate, will

be included in the study records, for illustrative purposes only. In addition, representative photographs of a normal littermate, will also be included in the study records, as needed and as appropriate, for comparison, where possible. **Representative photographs of all malformations with comparison photographs of normal fetuses will be included in the final report, for illustrative purposes only.** Prenatal data (viable and nonviable fetuses, early and late resorptions, pre- and post-implantation loss, and the fetal sex distribution) will be presented on a group mean basis and additionally as proportional data (% per litter).

15.2.1. External

Each viable fetus will be examined in detail, sexed, weighed, and euthanized by a subcutaneous injection of sodium pentobarbital in the scapular region. Nonviable fetuses (the degree of autolysis is minimal or absent) will be examined, crown-rump length measured, weighed, sexed and tagged individually. The crown-rump length of late resorptions (advanced degree of autolysis) will be measured, the degree of autolysis recorded, a gross external examination performed (if possible), and the tissue will be discarded.

15.2.2. Visceral (Internal)

Fetuses will be examined for visceral cardiac anomalies by dissection in the fresh (non-fixed) state. The thoracic cavity will be opened and dissected using a technique described by Stuckhardt and Poppe[8] with the exception that internal examination will be limited to a thorough examination of the heart and great and major blood vessels only. **Any observed ventricular septal defects will be categorized by size (<1 mm, 1 to 2 mm, or >2 mm) and location (muscular or membranous).** The abdomen will be opened with the sole purpose of internal confirmation of the sex of all fetuses. All carcasses will be discarded following completion of internal examination.

16. STATISTICAL METHODS

All analyses will be two-tailed for significance levels of 5% and 1%. All statistical tests will be performed using a computer with appropriate programming as referenced below. Data from nongravid females will be excluded from calculation of means and from comparative statistics. The litter, rather than the fetus, will be considered as the experimental unit.

Comparative statistics will not be performed on in-life or necropsy data from exposure assessment phase animals.

Data for the positive control substance group will be compared to the control group using a two-sample t-test[9] to determine intergroup differences.

16.1. Maternal In-Life Data

Continuous data variables (maternal body weights [absolute and net], body weight gains [absolute and net], food, and water consumption of each interval) will be subjected to a parametric one-way analysis of variance (ANOVA).[10] If the results of the ANOVA are

significant ($p<0.05$), Dunnett's test[11] will be applied to the data to compare the test substance treated groups to the control group.

16.2. Laparohysterectomy Data

The group mean numbers of corpora lutea, implantation sites, viable fetuses, maternal gravid uterine weights and mean fetal weight (separately by sex, and combined) will be subjected to a parametric one-way analysis of variance (ANOVA) and Dunnett's test as described above.[NOTEREF _Ref459642388 \h * MERGEFORMAT],[NOTEREF _Ref459642350 \h * MERGEFORMAT] The mean litter proportions of prenatal data (% per litter of viable and nonviable fetuses, early and late resorptions, total resorptions, pre- and post-implantation loss, and the fetal sex distribution) will be subjected to the Kruskal-Wallis nonparametric ANOVA test[12] to determine intergroup difference. If the results of the ANOVA are significant ($p<0.05$), Dunn's test[13] will be applied to the data to compare the test substance treated groups to the control group.

16.3. Fetal Morphology Data

The mean litter proportion (% per litter) of total fetal cardiac malformations and developmental variations and of each particular visceral cardiac malformation or variation will be tabulated. The mean litter proportions of fetal cardiac malformations and developmental variations will be subjected to the Kruskal-Wallis nonparametric ANOVA test followed by Dunn's test (if appropriate), to compare the test substance treated groups to the control group, as described above.[NOTEREF _Ref459642436 \h * MERGEFORMAT],[NOTEREF _Ref459642446 \h * MERGEFORMAT]

17. MAJOR COMPUTER SYSTEMS - DATA ACQUISITION, ANALYSIS, AND REPORTING

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by protocol, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by protocol and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

All computerized systems used for data collection during the conduct of this study have been validated (with the exception of Microsoft Office and GraphPad Prism® 2008); when a particular system has not satisfied all requirements, appropriate administration and procedural controls were implemented to assure the quality and integrity of the data. The actual version number will be specified in the report.

Critical Computerized Systems

Program/System	Description
Archive Management System (AMS)	In-house developed application for storage, maintenance, and retrieval of information for archived materials (e.g., lab books, study data, wet tissues, slides, etc.).

Program/System	Description
Bio Medic Data Systems (BMDS) Implantable Micro Identification™ (IMI-1000 or IMI-500)	Animal identification
Dionex Chromeleon® software, Varian MS Workstation® software, Agilent ChemStation® software, or Molecular Devices SpectraMax® software	Used for chromatographic data acquisition and quantitation
InSight® Publisher	Electronic publishing system (output is Adobe Acrobat, PDF).
Logbook™ ELN	System (Instem) used to document study events.
Master Schedule	Maintains the master schedule for the company.
MD5 Checksum Tool	Used to generate and verify MD5 checksums during the final report generation process to create a significant, permanent link between the electronic study report and the signature page.
Metasys DDC Electronic Environmental Control System	Controls and monitors animal room environmental conditions.
Microsoft Office 2010 or higher; GraphPad Prism® 2008	Used in conjunction with the publishing software to generate study reports.
Provantis Dispense™	Comprehensive system (Instem LSS Limited) to manage test materials, including receipt, formulation instructions, and accountability.
SAS®	Statistical (non-WTDMS™) analyses
Watson LIMS™	Laboratory Information Management System used for sample tracking, run planning, quantitation, and reporting results.
WIL Formulations Dispense System (WFDS)	In-house developed system for use in conjunction with Provantis Dispense™ to ensure proper storage and use of formulations.
WIL Metasys	In-house developed system used to record and report animal room environmental conditions.
WIL Toxicology Data Management System™ (WTDMS™)	In-house developed system used for collection and reporting of in-life and postmortem data.

Note: Version numbers of WTDMS™ programs used for the study are presented on the report data tables (reporting programs); version numbers and release dates are otherwise maintained in the study records and/or facility records.

18. AMENDMENTS AND DEVIATIONS

Changes to the approved protocol shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary protocol changes in advance with the Sponsor.

All protocol and SOP deviations will be documented in the study records. Deviations from the protocol and/or SOP related to the phase(s) of the study conducted at a Test Site shall be documented, acknowledged by the PI/IS, and reported to the Study Director for authorization/acknowledgement. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

19. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, electronic data, documentation, protocol, retained samples and specimens, and interim (if applicable) and final reports will be archived by no later than the date of final report issue. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least 1 year after issue of the Draft Report, the Sponsor will be contacted.

For work product shipped or generated by a test site, archiving will be conducted per test site SOPs and will be described in the test site report.

Unless otherwise indicated, any remaining clinical pathology, toxicokinetic, and/or analytical samples will not be archived, but will be discarded prior to issuance of the final report.

Any work product, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor to be shipped by Charles River to another location will be appropriately packaged and labeled as defined by Charles River SOPs and delivered to a common carrier for shipment. Charles River will not be responsible for shipment following delivery to the common carrier.

20. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft and Final Report provided in Adobe Acrobat PDF format (hyperlinked and searchable at final) along with a Microsoft Word version of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Testing Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation.

Reports should be finalized within 6 months of issue of the Audited Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Testing Facility unless other arrangements are made by the Sponsor.

21. ANIMAL WELFARE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (Code of Federal Regulations, Title 9), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* from the Office of Laboratory Animal Welfare,[14] and the *Guide for the Care and Use of Laboratory Animals* from the National Research Council.[15] The protocol and any amendments or procedures involving the care or use of animals in this study will be reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee before the initiation of such procedures.

If an animal is determined to be in overt pain/distress, or appears moribund and is beyond the point where recovery appears reasonable, the animal will be euthanized for humane reasons in accordance with the *American Veterinary Medical Association (AVMA) Guidelines on Euthanasia* and with the procedures outlined in the protocol.[16]

By approving this protocol, the Sponsor affirms that there are no acceptable non-animal alternatives for this study, and that it does not unnecessarily duplicate any previous experiments.

22. REFERENCES

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TESTING FACILITY APPROVAL

The signature below acknowledges Testing Facility Management's responsibility to the study as defined by the relevant GLP regulations.

_____Date:_____
Donald G. Stump, PhD, DABT
Senior Director, Toxicology
Testing Facility Management

The signature below indicates that the Study Director approves the study protocol.

_____Date:_____
Prägati Sawhney Coder, PhD, DABT
Director, Developmental and Reproductive Toxicology
Study Director

SPONSOR APPROVAL

The protocol was approved by the Sponsor by Email on 12-Apr-2018. The signature below confirms the approval of the protocol by the Sponsor Representative.

_____Date:_____

Christopher J. Bevan, PhD, DABT
Director, Scientific Programs
Halogenated Solvents Industry Alliance, Inc.
Sponsor Representative